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Anticancer Activity of Some Synthesized Combretastatin (CA) And Benzothiazole (BT) Derivatives

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Abstract

Compounds of CA-BT hybrids were synthesized and purified efficiently and characterized through NMR, C^{14} and HPLC studies. To explore the cytotoxic nature of synthesized chemical compounds, MCF-7 (breast carcinoma cell line) and K562 (leukemia cell line) were taken according to NCI based GI_{50} schedule. The MTT assay was also conducted to find out the cell viability. Compounds 7D, 7H, C1, C2 were identified as potent molecules showing the compounds 7H has quite good cytotoxic constantly in both the cell lines. Further experiment FACS was carried out to analyze the cell cycle so that effect of each compound could be understand easily. The tested synthetic conjugates showed G_2/M phase of the cell cycle arrest up to 50%. The G_2/M cell cycle seizure was attained through tubulin deformation and it was proved via confocal microscopy after immunostaining. Among all the compounds, 7D arrest G_2/M phase of cell cycle through the up regulation of cycline B1.

1. Introduction

Malignancy or cancer is presently next in highest rank after heart disease. Therefore, the necessity for novel anticancer therapeutics becoming more aggressive and needful. Tumor is generally regarded as irregular bulk tissue; whose development surpasses than normal tissue.

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

Even so, it resumes growth in the alike fashion after termination of the stimuli which have instigated it. The last two decades have perceived an outstanding transformation in the field of tumor chemotherapy [1,2].

The combretastatins belongs to an antimitotic group of agents generally extracted from the bark of the South African tree *Combretum caffrum* [3]. Cytoskeleton of the cell comprised of complex cellular network consists of microtubules which plays a key factor in retaining cell structure, particularly in the case of neovasculature. Undeveloped vasculature endothelial cells are more sensitive to anti-micro tubular agent molecules due to less developed actin cytoskeleton [4,5].

Effectiveness of benzothiazoles seems to be facilitated by selective metabolism of the medication in tumor cell lines, as only sensitive cells tends to gather and generate the foremost sedentary metabolite 6 hydroxybenzothiazole [6]. Literature review suggests that those benzothiazole compounds substituted at number 2 position have significant potential to unveil anticancer activity [7-12]. Broad preclinical and clinical researches produced some remarkable and fine technologies and drugs to control abnormal cell growth. However, they have been only successful in reducing the illness. Till now there are no particular anticancer or antitumour drug available which can limit the abnormal cell growth completely.

Every hour lapsed, numerous fresh bioactive compound or synthetic chemical objects have been screened. However, they never conclusively reach to the point of selection of drug status due significant adverse effects. Superlatively, if any drug claims to being considered acceptable that it must not express any adverse consequences on natural healthy cells. In an effort to decrease this adverse effects [13, 14]. Many fundamental alterations to CA-4 have been testified comprising alternation of the A and B-rings substituents [15,16,17]. However major variations of the B-ring results in reduced biological activity [17]. Novel structure drugs always need to be tested to determine their activity in cells metabolic pathway, as well as to predict the response to treatment of both the tumor and the patient. Hence we observe the anticancer activity of such benzothiazole and combretastatin derivatives against MCF-7(breast carcinoma cell line) as well as K562 (human immortalized myelogenous leukemia) through various in vitro experiments sequentially.

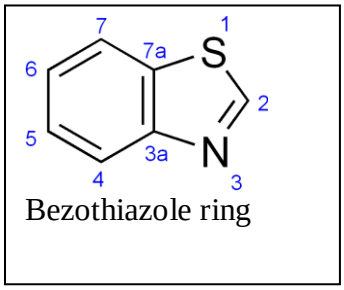
ISSN: 0971-1260
Vol-22-Issue-17-September-2019

2. Materials and methods

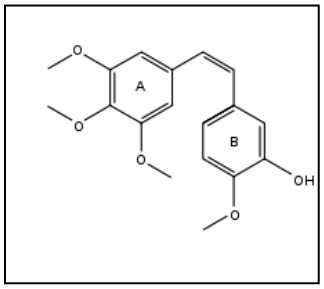
2.1.

Sl No.	Compound Name	MW(kd)
1.	AKL-CA4(+ve control)	316.3500
2.	AKL-C1(SM1)	244.2900
3.	AKL-C2(SM2)	316.3700
4.	AKL-4c	419.4465
5.	AKL-6d	473.5000
6.	AKL-6e	473.5000
7.	AKL-6f	503.6000
8.	AKL-7d	503.6000
9.	AKL-7j	488.6000
10.	AKL-7h	458.5322

*From 4-10 combretastatin derivative, *1 is CA4 combretastatin, *2 and 3 are benzothiazole



Bezothiazole ring



2.2. Cell Culture:

MCF-7 (a breast carcinoma cell line) and K562 (first human immortalized myelogenous leukemia) were used in this study. These cells are incubated in RPMI medium supplemented with 10% fetal calf serum, 100µg/ml penicillin-G and 100µg/ml streptomycin sulfate. This medium works in a 5% carbon dioxide atmosphere. RPMI 1640 uses a bicarbonate buffering system with an initial pH 8 formulation [18].

2.3. MTT cell viability assay:

MCF-7 and K562 cells were cultured for overnight. Cell treatment was doen with AKL-CA4 (+ve control), AKL-C1(SM1), AKL-C2(SM2), AKL-4C, AKL-6d, AKL-6e, AKL-6f, AKL-7d, AKL-7j, and AKL-7h, at a concentration of 1µM, 2µM, 4µM, and 8µM followed by an Incubation for 24 hrs. Growth media was decanted and 10 µL MTT dye was added followed by

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

an incubation at 36 °C for 120 minutes. The resultant material was dissolved in 150 µL extraction buffer. OD was taken at 570 nm [19].

2.4. Cell cycle analysis by Flow Cytometry

5 × 105 MCF-7 and K562 cells were seeded in 60 mm dish for 24h growth. 2 µM concentrations of AKL-CA4 (positive control), AKL-C1 (SM1), AKL-C2 (SM2), AKL-4C, AKL-6d, AKL-6e, AKL-6f, AKL-7d, and AKL-7j, and AKL-7 h, compounds were tested for 25h. Cells were harvested with Trypsin-EDTA followed by fixation with chilled 70% ethanol at 4°C for a time period of 30 minute. PBS wash was and incubation with 1 mg/mL RNAase solution at 36 °C for 0.5h was done consecutively. Tested cells have been harvested via centrifugation at 2100 rpm for 6 minute. The cells were stained by 300 µL of DNA staining mixture. Cells were measured by flow cytometer and histograms were analyzed using Summit Software [20].

2.5. Protein extraction and quantification

After analysis of MTT assay result and FACS result, four compounds are selected for further experiments including the positive control against the MCF-7 cell line as the tested compound exhibited better effectiveness than upon K562 cell line. Hence, 5 × 100 MCF-7 cells were cultured in 0.6 cm dish for 24h, 2 µM concentration of AKL-CA4, AKL-C2(SM2), AKL-7d, and AKL-7h compounds were added and again incubated for 24 h. Total cell lysates was obtained with ice-cold RIPA buffer. After centrifugation protein content was determined by Bradford method [7, 20].

2.6. Western Blots analysis

Standard western blot procedure was adopted after 12% SDS PAGE followed by Mouse monoclonal antibodies cyclineB1, p21, Cdk1 and Rabbit polyclonal PARP (poly ADP ribose polymerase) were used (IMGENEX India and Millipore company Ltd). Rabbit polyclonal antibodies such as β-actin, α tubulin were used from IMGENEX India and Santacruz Biotech Company.

2.7. Immunofluorescence study for Tubulin polymerization by confocal microscope

MCF-7 cells were culture in similar fashion as described before to test AKL-7h of 2µM intensity. Cells were fixed with 5% paraformaldehyde, 0.03% glutaraldehyde in PBS and permeabilized by

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

with 100% methanol (−20 °C). Primary antitubulin (mouse monoclonal) antibody followed by FITC conjugated auxiliary Rat Anti-IgG antibody were used and observed under confocal microscope outfitted with FITC settings. Nocodazole (Noc) (5μM) was used as positive control [20].

3. Result and discussion

3.1. In vitro cytotoxicity:

The anticancer activity of those conjugates, carried out against MCF-7 & K562 cell lines shows significant results maximum by AKL-CA4(positive control), AKL-C2, AKL-7D, AKL-7H at a concentration of 2μM (Table 1, 2 and Figure 1, 2).

concentration (μM)	Akl-CA4	Akl-C1	Akl-C2	Akl-4C	Akl-6D	Akl-6E	Akl-6F	Akl-7D	Akl-7J	Akl-7h
1	**0.472**	**0.806**	**0.9**	**1.031**	**1.762**	**1.951**	**1.082**	**0.783**	**1.876**	**1.79**
2	**0.329**	**0.262**	**0.4**	**0.226**	**0.345**	**0.439**	**0.428**	**0.317**	**0.232**	**0.625**
4	**0.808**	**0.725**	**0.974**	**0.776**	**1.036**	**0.955**	**0.884**	**0.458**	**0.885**	**1.221**
8	**0.368**	**0.197**	**0.63**	**0.154**	**0.208**	**0.344**	**0.181**	**0.131**	**0.188**	**0.201**

concentration (μM)	Akl-CA4	Akl-C1	Akl-C2	Akl-4C	Akl-6D	Akl-6E	Akl-6F	Akl-7D	Akl-7J	Akl-7h
1	0.549	1.278	0.889	1.246	0.9	1.283	1.087	0.856	0.679	0.596
2	0.54	0.546	0.284	0.351	0.367	0.515	0.588	0.254	0.304	0.441

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

4	1.379	1.435	1.417	1.267	1.38	1.037	0.782	0.961	0.552	0.673

Tab1: control-0.903(With DMSO same as sample volume), MCF-7 cell line
Tab2: control-1.559(With DMSO same as sample volume), K562 cell line

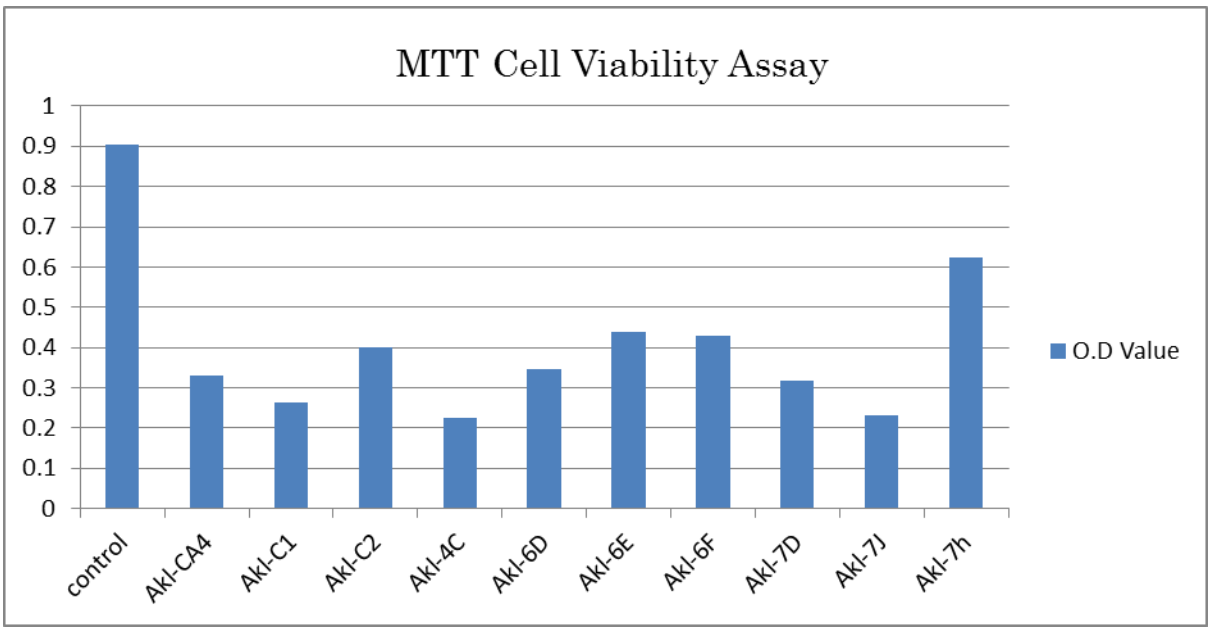


Figure 1: O.D values against cell line MCF-7 at 2µM concentration

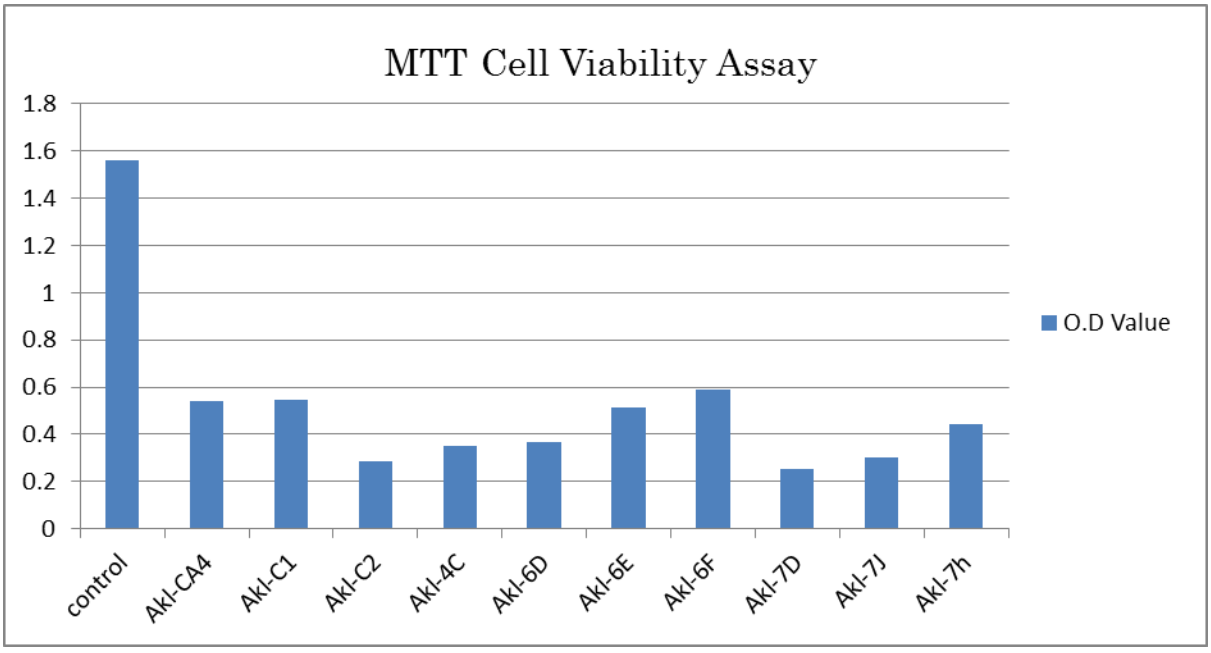


Figure 2: O.D values against cell line K562 at 2µM concentration

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

The compoundsC1, C2, 7D were found to the highly cytotoxic in equally in MCF-7 and K562. But 7D shown consistency with regard to cytotoxic nature in both the cell line. Thus it was considered of our interest for future studies.

Effect on cell cycle

compounds	G0	G1	S	G2/M
Control	7.18	65.50	10.80	16.52
CA4	31.76	60.18	3.65	4.41
C1	2.90	65.03	10.60	21.44
C2	2.70	62.17	11.59	23.54
4C	4.02	67.75	10.6	18.12
6D	6.48	60.62	11.0	21.90
6E	5.77	62.06	10.49	21.68
6F	17.54	53.15	8.35	20.96
7D	2.66	62.14	11.35	23.85
7H	4.74	62.26	9.97	23.03
7J	5.83	65.29	10.19	18.69

Table 3: Cell division phases of MCF-7 at 2µM test sample concentration

compounds	G0	G1	S	G2/M
Control	4.53	76.34	8.21	11.68
CA4	30.14	67.37	0.82	1.72
C1	2.83	74.95	8.49	14.62
C2	4.7	72.41	7.40	16.12
4C	5.14	73.56	7.21	14.84
6D	6.32	72.25	7.30	14.85
6E	5.34	75.19	7.29	12.89
6F	5.17	74.85	5.96	14.58
7D	4.00	73.24	7.03	16.36
7H	6.18	71.0	7.46	15.95
7J	6.99	74.71	6.25	12.49

Table 4: Cell division phases of K562 at 2µM test sample concentration

Furthermore, to check whether the compounds have any effect upon cell cycle distribution, FACS analysis was conducted in both MCF-7 and K562 cell lines. Both the cell lines found to

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

exhibit G2/M cell cycle arrest with the CA-BT based hybrid compounds. But MCF-7 cells were found to slightly more effective in case of G2/M cell cycle arrest.

In MCF-7 cell line control cells shown 16.52% G2/M but CA4, C1, C2, 4C, 6D, 6E, 6F, 7D, 7H and 7J compound treated cells shown 4.41%, 21.44%, 23.54%, 18.12%, 21.90%, 21.68%, 20.96%, 23.85%, 23.03% and 18.69% G2/M respectively. Hence 7D and 7H is showing most effective results in cell cycle distribution. Similarly in K562 cell line control cells shown 11.68% G2/M but CA4, C1, C2, 4C, 6D, 6E, 6F, 7D, 7H and 7J compound treated cells shown 1.72%, 14.62%, 16.12%, 14.84%, 14.85%, 12.89%, 14.58%, 16.36%, 15.95% and 12.49% G2/M respectively. Hence here also 7D and 7H is showing most effective results in cell cycle distribution.

3.2. Effect on Expression of cycline B1 and cdk1

CyclineB1 and cdk1 levels were examined in tested cell line that were treated with AKL-C2, AKL-7d and AKL-7H with positive control. cyclin-B1 is a protein that in humans is encoded by the CCNB1 gene and it is mainly involved in mitosis. Cyclin dependent kinase 1 also known as Cdk1 or cell division control protein 2 homolog is a highly conserved protein that functions as a serine/threonine kinase, and is a key player in cell cycle regulation. Western blot results indicated that there was reduction of cyclineB1 (Figure 3) in case of C2 more than CA4 and 7D. In case of 7H, it was increased. On the other hand, cdk1 is also slightly reduced at C2 but increased in CA4, 7D and 7H (Figure 3).

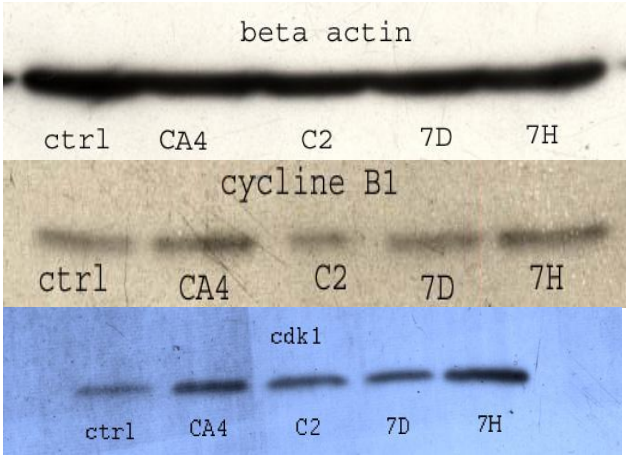


Figure 3: Effect of CA-BT hybrids on the expression of cycline B1 and cdk1, β actin used as loading control

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

3.3. Effect on expression of PARP(cleaved) and p21

Poly [ADP-ribose] polymerase 1 (PARP-1) also known as NAD+ ADP-ribosyltransferase 1 or poly[ADP-ribose] synthase 1 is generally encoded by the PARP1 gene. It is conjectured that PARP1 inhibitors might verify extremely operative therapies for carcinoma with low adverse effects and selective pathway. P21 is considered important in the regulation of apoptotic pathway induced by various stimuli. Western blot analysis shows that PARP is significantly decreased in case of CA4, C2 and 7D. On the other hand, p21 slightly decreased in case of 7D and 7H but increased in CA4 and C2.

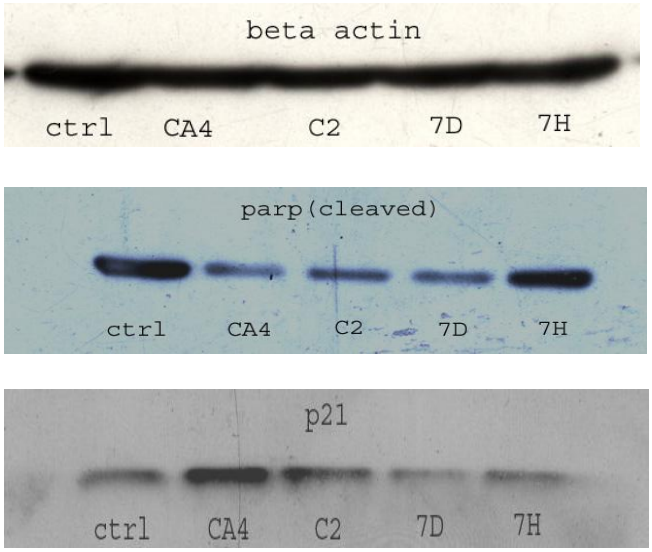


Figure 4: Effect of CA-BT hybrids on the expression of PARP (cleaved) and p21, β actin used as loading control

3.4. Effect on tubulin polymerization

The reason behind this study was to check the effect of tested drug whether it is interfering with the spindle development in the stage of mitosis and DNA differentiation or not. Therefore, these compounds were assessed for the negative effect upon tubulin web fiber construction at 2 µmol for 24h by taking Nocodazole as a standard and DMSO control on tubulin web fiber extracted from MCF-7 cells. After 24 hrs 7H treated cell was stained as described in material section and was used for microscopy. Analysis of the results showed that interference of microtubule web fiber was affected expressively in compound 7H shown in (Figure 5). From the MTT cell viability assay we can see 7D and 7H are most effective on both MCF-7 and K562 cell lines but simultaneously positive control (CA4) and C2 are effective also. Further analysis by flow

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

cytometry confirmed the above statement through G2/M phase arrest in the cell cycle after treating the cell lines with those compounds in a concentration at 2µM.

Hence CA4, C2, 4C, 7D and 7H compounds are selected for further analysis through western blotting on MCF-7cell line to observe whether the compounds have any effect upon proteins related to cell cycle regulation or not. There was reduction of cyclineB1 (Fig3) in case of C2 more than CA4 and 7D. In case of 7H, it was increased. On the other hand cdk1 is also slightly reduced at C2 but increased in CA4, 7D and 7H (Fig3). On another set of experiment shows that PARP is significantly decreased in case of CA4, C2 and 7D. On the other hand p21 slightly decreased in case of 7D and 7H but increased in CA4 and C2. The western blot result indicates 7D and 7H are most constant effect on expression of proteins related to cell cycle regulation via increasing or decreasing tubulin compactness and amount.

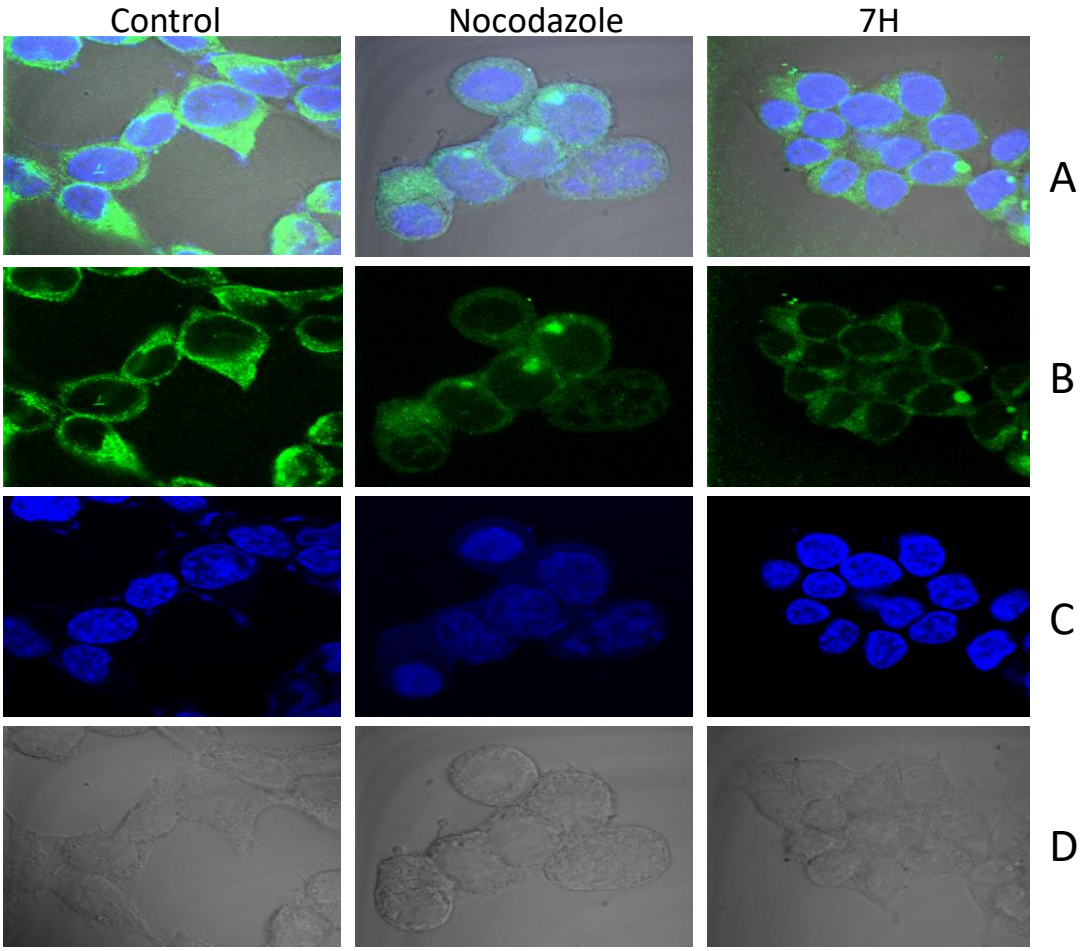


Fig5:Effect of Compound 7H on Tubulin polymerization on MCF-7 cell line; A:Merged ; B:Tubulin; C: DAPI; D: DIC

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

4. Conclusion

In conclusion, we have here described some anti-cancerous benzothiazole and combretastatin derivative compounds, which showed significant cytotoxic activity towards human cancer cell lines tested like MCF-7 (breast carcinoma cell line) and K562 (human immortalized myelogenous leukemia line). The molecular level or pathways of cancer cell death were investigated through Flow cytometry, Western blotting and Immunostaining. Further experimental investigation for benzothiazole and combretastatin is needed to develop anticancer drugs or biomarkers in pharmaceutical, biotech industries etc.

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ISSN: 0971-1260
Vol-22-Issue-17-September-2019

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ISSN: 0971-1260
Vol-22-Issue-17-September-2019

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